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Cell surface properties of Phenol-Utilizing Bacteria Isolated from petroleum refinery wastewater

Authors:
Nwanyanwu CE[1], Alisi CS[2], Nweke CO[1] and Orji JC[1].

Institution:
1. Department of Microbiology, Federal University of Technology, P.M.B. 1526, Owerri, Nigeria.

2. Department of Biochemistry, Federal university of Technology, P.M.B. 1526, Owerri, Nigeria.

ABSTRACT:

Cell surface hydrophobicity of six phenol-utilizing bacteria isolated from Port Harcourt Petroleum refinery wastewater was assessed via bacterial adhesion to hydrocarbon (BATH), salt aggregation test (SAT) and Congo red binding (CRB) assays. The test organisms exhibited high to moderate hydrophobicity with BATH assay respectively when *n-octane* and *p-xylene* were employed. *Bacillus* sp. RBD, *Escherichia coli.* OPWW, *Corynebacterium sp.* DP, *Citrobacter sp.* RW and *Pseudomonas sp.* SD showed moderate hydrophobicity in SAT assay. On the other hand, *Pseudomonas sp.* RWW showed high hydrophobicity in SAT assay. Similar results of moderate hydrophobicity were obtained with CRB except *Corynebacterium sp.* DP that exhibited high hydrophobicity value of 14.70±1.00µg. The results obtained in this study showed that the isolates are mainly moderately hydrophobic which make them good candidates in the clean up activity of organic pollutants in polluted sites.

Keywords:
Phenol-utilizing bacteria, Hydrophobicity, SAT, Congo red binding.

Corresponding author:
Nwanyanwu CE.

Email:
cnwanyanwu2000@yahoo.com

Web Address:
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INTRODUCTION

Large amounts of water are used in the petroleum refinery activity and, consequently, significant volumes of wastewater are generated reaching about 0.4-1.6 times the volume of processed oil (Coelho *et al.,* 2006). The wastewater generated by the refinery is characterized by the presence of organic and inorganic pollutants that gave it its toxic nature. The toxic effects of refinery effluents to bacteria have been reported (Nwanyanwu and Abu, 2010; Krishnakumar *et al.,* 2007). Bacteria are unable to insulate themselves from the toxic nature of their habitat because of their large surface area that is exposed to these harsh environments. These pollutants in the effluent exert their toxic effects on the bacterial surfaces thereby inhibiting their attachment to substrates and other surfaces. The most important mechanism of toxic action of these pollutants is the destabilization of cell membrane (Walsh *et al.,* 2003). This effect results in adaptation and changes in effluent autochthonous microbial physiological functions and moreso can have serious impact on the cell surface hydrophobicity and biodegradation processes (Nwanyanwu *et al.,* 2012; Kaczorek *et al.,* 2008; Leung *et al.,* 1997)

. The hydrophobicity of the outermost bacterial surface has been cited as a factor in partitioning of microorganisms at the air–water interface and in the adherence of bacteria to wide variety of surfaces as well as growth of cells on insoluble hydrophobic substrates such as hydrocarbons (Rosenberg, 1981). Cell surface hydrophobicity has been found to be involved in interfacial interactions of microbial cells with other microbial cells (flocculation) and with air (flotation) (Rosenberg and Kjelleberg, 1986). The hydrophobic nature of bacterial surfaces has long been blamed for the formation of stable foams in wastewater treatment plants. Hydrophobicity appears to be imparted by different chemical components of the cell wall in different bacteria; these components include lipoteichoic acids and proteins.

The utilization of hydrocarbon and other aromatic pollutants found in wastewater treatment plants are aided by contact between hydrophobic organic compounds and the cells. The ability to adhere on to hydrocarbon is correlated with cell surface hydrophobicity. Many types of microorganisms are found in wastewater treatment plants. Hydrophobic microorganisms, in particular, are capable of adhering to the oil-water interface thereby utilizing oil components as well as other organic compounds in wastewater treatment plants as a source of energy for growth and metabolism. This process is of great interest for the preservation of the natural environment by reducing the amount of oil-related contaminants (Pijanowska *et al.,* 2007).

Not much work has been done to assess the cell surface hydrophobicity of bacteria strains isolated from refinery effluent treatment plants. This study is therefore aimed at investigating the cell surface hydrophobicity of bacteria strains found in Port Harcourt petroleum refinery effluent treatment plants and water body receiving petroleum refinery effluent.

MATERIALS AND METHODS

Sample collection and characterization

Physicochemically treated raw wastewater (addition of additives, flocculation, sedimentation and filtration) (RWW), biologically treated wastewater (Rotary biodisk) (RBD), observation pond treated wastewater (oxidation pond) (OPWW) and discharge pipe wastewater (DP) samples of Port Harcourt petroleum refinery were collected in sterile polyethylene containers. Also river water (RW) and sediment (SD) samples from Okrika River in Port Harcourt Nigeria that receives the petroleum refinery wastewater were collected. The containers were rinsed twice with the samples at the point of collection. To avoid deterioration, the samples were taken to the laboratory in icebox within 6h of collection for analyses. pH, biological oxygen

demand (BOD), chemical oxygen demand (COD), phosphate (PO_4) and sulphate (SO_4) were determined according to APHA (1985) whereas lead (Pb), zinc (Zn), copper (Cu) were determined by atomic absorption spectrophotometer (Perkins Elmer 3110) respectively. Oil and grease of the samples were determined using partition-gravimetric method (Noweco, 1997). Phenol content was determined as described by Folsom *et al*., (1990). Electrical conductivity was measured with electrical conductivity meter (HACH conductivity meter).

Isolation and identification of bacterial strains

Phenol-utilizing bacteria were isolated from the samples by spreading 0.1ml of decimally diluted (10-4) wastewater samples on mineral salt agar plates amended with 2.5mM phenol as described by Hill and Robinson (1975). The medium has the following composition (mg/l) of KH_2PO_4, 840; K_2HPO_4, 750; $(NH_4)_2SO_4$, 400; $MgSO_4.7H_2O$, 60; NaCl, 60; $CaCl_2$, 60; $FeCl_3$, 60 and 15 g agar added to solidify the medium. Ketoconazole at 50µg/ml was added to the medium to exclude fungi and pH adjusted to 7.2. The plates were incubated for 72 h and the developed colonies were purified on freshly prepared nutrient agar plates. The purified isolates were characterized biochemically following standard microbiological methods. Identification to the genus level followed the schemes of Holt *et al.,* (1994). The isolates were maintained on nutrient agar slants.

Preparation of inoculum and culture condition

The bacterial isolates for the assay were grown in 100 ml of Bushnell–Haas (BH) broth consisting of (g/l): KH_2PO_4, 1.0; K_2HPO_4, 1.0; NH_4NO_3, 1.0; $MgSO_4{\cdot}7H_2O$, 0.2; $FeCl_3{\cdot}6H_2O$, 0.085; $CaCl_2{\cdot}2H_2O$, 0.02 and phenol, 0.05 contained in 250 ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker (150 rpm) for 24 h at room temperature (28±2^{o}C). The cells were recovered by centrifugation (6,000 rpm for 10 min) and washed twice in phosphate buffered saline (PBS, 0.02M; pH 7.2 for BATH assay and Congo red assay and pH 6.8 for SAT). The washed cells were resuspended in the buffer medium and the turbidity adjusted spectrophotometrically to give an optical density of 1.0 at 540nm.

Cell surface hydrophobicity assays

Cell surface hydrophobicity of the phenol-utilizing bacteria was assessed using the bacterial adherence to hydrocarbon (BATH), modified salt aggregation test (SAT) and Congo red binding.

Bacterial adherence to hydrocarbon

BATH was performed as described by Rosenberg *et al*., (1984). The cell suspensions were dispensed in 4ml aliquots into sterile 20ml screw capped culture tubes. The tubes received different volumes viz 0.1, 0.2, 0.3, 0.4 and 0.5ml of either n-octane or *p-xylene* (Sigma Chemical Co., St. Louis, Mo., USA). The mixtures were vortexed for 2 min and allowed to stand for 15 min for the completion of biphasic formation. After phase separation, the aqueous phase was carefully recovered and the OD_{540} was determined (A1). Values were then expressed as the percentage of bacteria adhering to the hydrocarbons (A) compared with the control suspension (A0) as follows:

$$A\ (\%) = \frac{(A_0 - A_1)}{A_0} \times 100$$

The reference value for the BATH assay is the percentage of bacteria from 4ml of suspension that partition into 0.5ml of *n-octane* or *p-xylene.* Strains were considered strongly hydrophobic when values were >60%, moderately hydrophobic when values were in the range of 40 - 60% and hydrophilic when values were <40% (Basson *et al.,* 2008).

Salt aggregation test

The SAT assay was carried out as described by Lindahl *et al.,* (1981) with little modifications. The assay is based on bacterial precipitation in the presence of salts. Isolates were salted out (aggregated) by combining 25µl volumes of the cell suspension with equal (25µl)

volumes of a series of varying molarities (M) of $(NH_4)_2SO_4$ solution (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8, 3.0 and 4.0M) in different wells in a microplate. Addition of 400 µl of 0.1% w/v methylene blue solution to 10ml volumes of $(NH_4)_2SO_4$ solution facilitates better visualization of aggregation (Rozgonyi *et al.,* 1985). The plate was rocked for 4 min after which it was visually examined and scored against a white background for cell aggregation. Concentration of $(NH_4)_2SO_4$ solution causing aggregation was considered positive whereas the absence of aggregation was considered as negative. Hydrophobicity was expressed as the lowest salt concentration in the mixture that produced visual clumping. Classification was expressed as: < 1.0 M = strongly hydrophobic, 1.0-2.0 M=Hydrophobic, > 2.0 M = Hydrophilic.

Congo red binding assay

This assay is used to study the pigment binding ability of the strains as well as a marker of hydrophobicity. The experiment was performed as described by Qadri *et al.,* (1988). Aliquot (1.0ml) of bacterial suspension were transferred into screw capped test tubes containing 4ml of PBS amended with 25µg/ml of congo red dye and were incubated at room temperature for 15min. Thereafter, the Congo red bound to cells are removed by centrifugation at 6,000 rpm for 10min. The supernatant (cell free Congo red solution) was collected in separate tubes and its absorbance determined spectrophotometrically at 480nm against a PBS blank. The amount of congo red dye that bind to the cells were calculated from a standard curve as the difference between the amount added to the mixture and the amount remaining in the cell free Congo red solution. Uptake of Congo red greater than 10 µg was scored as strongly hydrophobic (Payne and Finkelstein, 1977).

RESULTS AND DISCUSSION

The physicochemical properties of the petroleum refinery wastewaters and Okrika river water and sediment are shown in Table 1. The level of oil and grease, Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD) and phenol content of the petroleum refinery wastewater samples are indication that the samples are polluted with organic compounds. This also showed that autochthonous microorganisms isolated from these samples are subjected to pollutant stress (Sarala and Sabitha, 2012). The organisms isolated from the refinery wastewater samples include *Pseudomonas sp.* RWW, *Bacillus sp.* RBD, *Escherichia coli* OPWW and *Corynebacterium sp.* DP while *Citrobacter sp.* RW and *Pseudomonas sp.* SD were isolated from Okrika river water and sediment samples

Table 1 Characteristics of the petroleum refinery wastewater and okrika river

Parameter/unit	Sample source					
	RWW	RBD	OPWW	DP	RW	SD
pH	7.64	8.18	7.45	8.87	8.97	6.80
Temperature (^{o}c)	26.4	26.1	26.8	26.7	-	-
Electrical Conductivity.(µs/cm)	845	443	926	643	364	615
Oil and grease (mg/l)	17.5	15.0	21.0	16.0	16.0	100
BOD (mg/l)	32.0	8.0	12.8	12.8	-	-
COD (mg/l)	112.0	76.0	114.0	84.0	-	-
PO_4 (mg/l)	0.22	0.14	0.13	0.12	0.12	0.07
SO_4 (mg/l)	37.63	13.52	35.3	11.8	117	115
Phenol (mg/l)	71.2	13.6	10.1	9.4	8.6	15.5
Pb (mg/l)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Zn (mg/l)	0.13	0.02	0.06	0.08	0.05	38.6
Cu (mg/l)	<0.01	<0.01	0.01	0.01	<0.01	0.061

Key : RWW = Raw wastewater, RBD = Rotary biodisk OPWW = Observation pond wastewater, DP = Discharge pipe RW = River water, SD = Sediment

respectively. The bacterial strains represent the preponderant morphotypes in their respective sources. These bacteria have been reported to grow on hydrocarbon and aromatic compounds as well as other organic pollutants in the environment (Akpoveta *et al.,* 2011; Okerentugba and Ezeronye, 2003; Mo *et al.,* 2000; Zhang and Miller, 1994)

Different results were obtained, depending on the method adopted to estimate cell surface hydrophobicity of the organisms. Cell surface hydrophobicity using BATH was evaluated in terms of change in absorbance at 540 nm. Figure 1 showed a progressive increase in the adhesion to hydrocarbon with increasing volume of *n-octane* and *p-xylene*. This indicated that the cells were partitioned into the hydrocarbon phase and the cells exhibited relative high surface hydrophobicity in the BATH assay. The loss of bacteria from the aqueous phase was approximately proportional to the volume of n-octane and p-xylene added to the cell suspension. The values from the BATH assay recorded for the partitioning of the organisms is the percentage of bacteria suspension that partitioned into 0.5ml of *n-octane* and *p-xylene* as indicated in Table 2. Sorongon *et al.,* (1991) and Lachica and Zink (1984) reported that loss of bacteria from aqueous phase was a function of hydrocarbon concentration employed. The organisms partitioned more in n-octane than in *p-xylene* as shown in Table 2. This may be as a result of devastating effect of *p-xylene* on the surface of the organisms than n-octane.

The adherence of the organisms correlated with volume of hydrocarbons with R2 values greater than 0.90 ($0.9044 \leq R2 \leq 0.9922$) for n-octane and greater than 0.94 ($0.9455 \leq R2 \leq 0.9795$) for *p-xylene* in all the bacterial strains (Figure 2). The high R2 values observed in all the bacterial strains indicated that volume of hydrocarbon was a strong determinant of the adherence. Using *n-octane* in BATH assay, *Pseudomonas sp.* RWW, *Bacillus sp.* RBD and *Escherichia sp.* OPWW are classified as strongly hydrophobic while *Corynebacterium sp.* DP, *Citrobacter sp.* RW and *Pseudomonas sp.* SD are moderately hydrophobic. Similarly, *p-xylene* indicated that all the organisms are moderately hydrophobic.

Cell surface hydrophobocity estimated by salt aggregation test (SAT) assay showed that majority of the test isolates aggregated at 1.0M $(NH_4)_2SO_4$ (Table 2). This indicated that the bacterial strains found in Port Harcourt refinery effluent are moderately hydrophobic. The differences in SAT values between *Pseudomonas sp*. RWW that showed hydrophobic and other test organisms that showed moderate SAT values may be as a result of differences on cell surface charges. This indicates that SAT values may be dependent on the charge on microbial surface as well as the age of the culture. This is in agreement with the results obtained by Qadri *et al.,* (1988) in which they found that aggregation of bacterial strains increase with old cultures as charges on microbial surfaces increases.

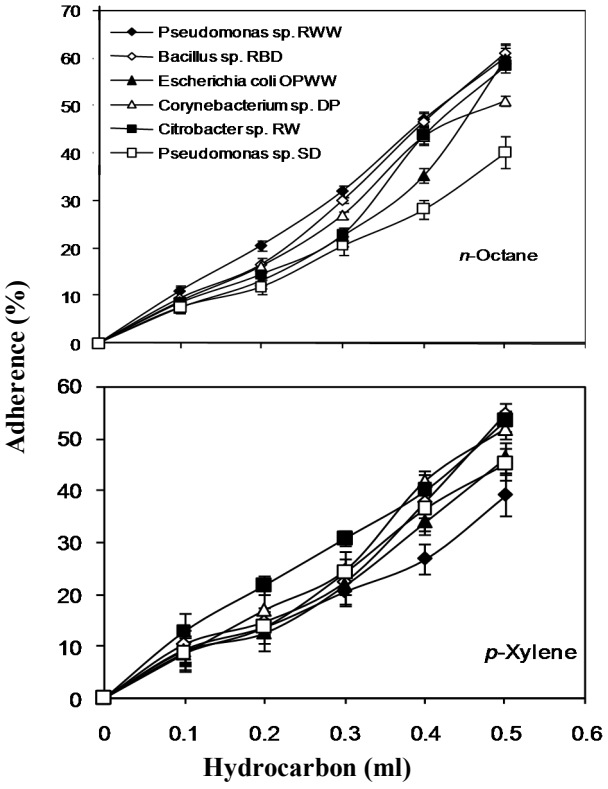

Figure 1: Effect of hydrocarbon and aqueous phase ratios on hydrophobicity of bacteria

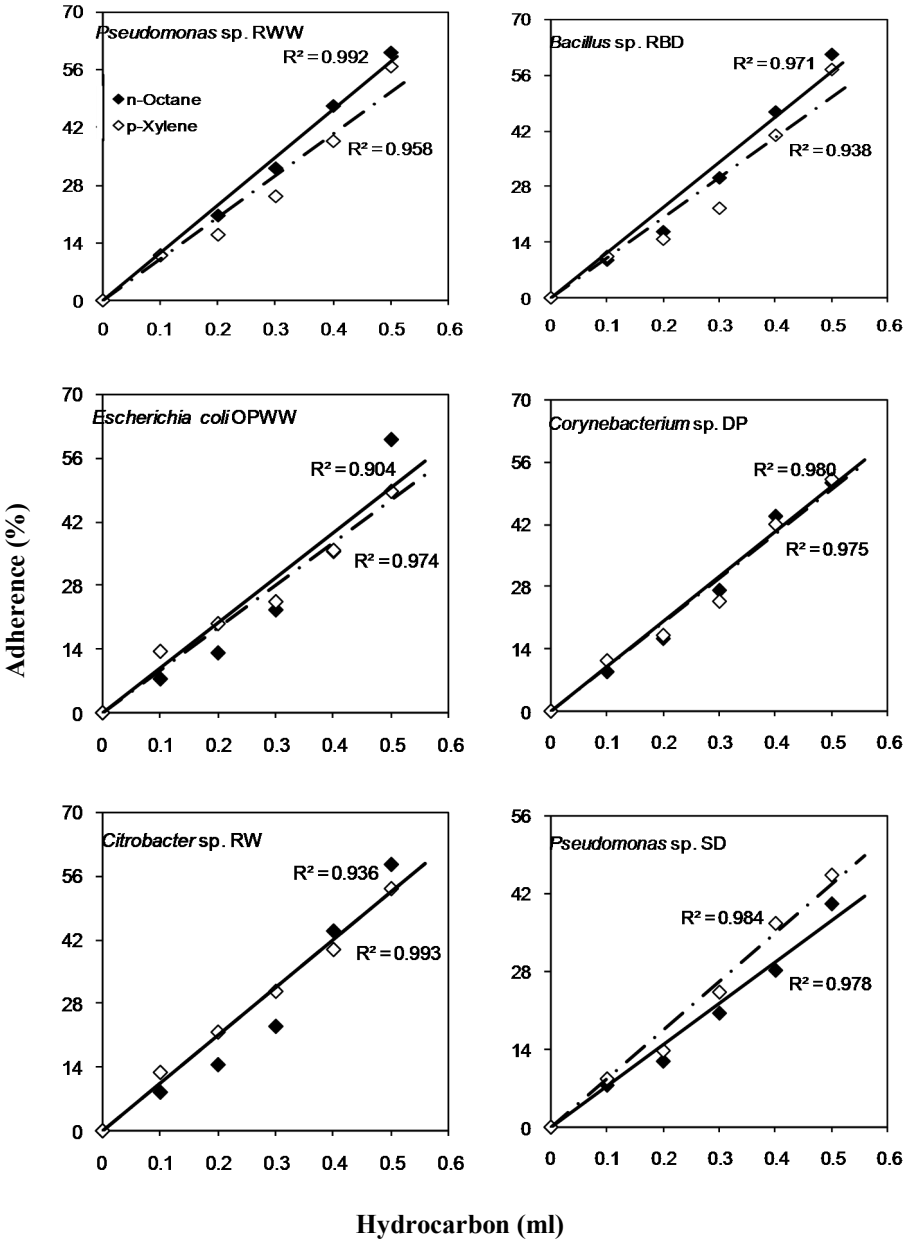

Figure 2: Correlation of hydrocarbon *(octane/xylene)* volume with percentage of adherence in cell surface hydrophobicity of The bacteria. The solid line *(n-octane)* and dotted line *(p-xylene)* are predicted adherence values. The closed and open squares represent data obtained from *n-Octane* and *p-Xylene* respectively

The Congo red binding, commonly used as a marker of hydrophobicity (Majtan and Majtanova, 2001) was employed in this test and the test organisms exhibited distinct uptake of congo red in solution (Table 2). Previous studies (Haider *et al.,* 1990; Qadri *et al.,* 1988) have shown that congo red binding may reflect an arrangement of cell surface components. *Corynebacterium sp.* DP showed highest Congo red uptake of 14µg while the least Congo red uptake of 10.5µg was recorded for *Escherichia coli.* OPWW. Congo red binding test indicated that all the test organisms are strongly hydrophobic.

BATH and SAT hydrophobicity tests sometimes fail to correlate and this has also been observed in the

Table 2 Hydrophobicity characteristics of phenol-utilizing bacterial strains

Bacteria	% Adherence		SAT M $(NH_4)_2SO_4$	Congo red binding (µg)
	n-octane	*p*-xylene		
Pseudomonas sp. RWW	60.33 ± 2.59	56.78 ± 5.03	0.40 ± 0.00	11.80 ± 1.00
Bacillus sp. RBD	61.33 ± 1.92	54.83 ± 2.00	1.40 ± 0.00	10.90 ± 0.00
Escherichia coli. OPWW	60.06 ± 2.34	46.27 ± 2.87	2.00 ± 0.00	10.50 ± 5.00
Corynebacterium sp. DP	51.22 ± 1.02	51.94 ± 2.17	1.60 ± 0.00	14.70 ± 1.00
Citrobacter sp. RW	53.44 ± 1.95	53.44 ± 1.05	1.20 ± 0.00	12.10 ± 2.00
Pseudomonas sp. SD	45.22 ± 3.04	45.22 ± 3.07	1.00 ± 0.00	12.40 ± 3.00

BATH expressed as the percentage of bacteria from 4 ml of cell suspension that partition into 0.5 ml of *n-octane* or *P-xylene*. SAT expressed as the lowest $M(NH_4)_2SO_4$ concentration in the reaction mixture that produced visual clumping Uptake of Congo red dye greater than 10µg was scored as strongly hydrophobic

present study. Lack of correlation between SAT and BATH test results had been observed by Soto-Rodriguez *et al.,* (2003) and Lee and Yii (1996). Also the BATH and CRB, SAT and CRB sometimes fail to correlate with one another. Even within BATH assay using different hydrocarbons n-octane and p-xylene the hydrophobicity did not correlate as shown in Table 2. *Pseudomonas sp.* RWW, *Bacillus sp.* RBD and *Escherichia coli.* OPWW have relatively higher adherence to *n-Octane* than *p-Xylene*. The failure of BATH and SAT to correlate might be explained by the SAT assay measuring the hydrophobicity of the outer surface as a whole while the BATH assay measured it in terms of adhesion (Mattos-Guaraldi *et al.,* 1999). In addition, hydrophibicity and surface charge of bacteria may differ between species, strains, and changes with variation in physiological state of cells and composition of suspension media or might involve variable expression of surface-associated proteins between strains. Collectively, this might account for the diversity of BATH, SAT and CRB indices obtained in this work.

A cell surface hydrophobicity influences the direct contact of cell with hydrocarbons and is hence, one of the major factors affecting the degradation of organic compounds. Bioavailability of organic compounds, a physicochemical parameter critical in the overall rate of degradation, is a function of phase solubility and solution transport processes, hence, phenol is therefore, easily biodegraded. Therefore, phenol degrading bacteria with hydrophobic cell surface properties could be potentially applicable in the treatment of phenolic wastewater contaminated with hydrocarbons.

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